Role of the intracellular domain of the β subunit in Na,K pump function

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Abstract

The catalytic α subunit of the (Na,K)- and (H,K)-ATPases needs to be coexpressed with a β subunit in order to produce cation transport activity. Although the isoform of the β subunit is known to influence the functional characteristics of the Na,K pump, the role of the different domains of the β subunit is not fully understood. We have studied the function of a Na,K pump resulting from the expression of a wild-type α subunit with a N-terminally truncated mutant of the β subunit using the two-electrode voltage clamp and the cut-open oocyte techniques. While the maximal activity, measured as the K^+-activated outward current, was not significantly altered, the β N-terminal truncation induced an ouabain-sensitive conductance in the absence of extracellular K^+. The voltage dependence of the ouabain-sensitive charge distribution indicated that in the Na/Na exchange conditions, the E1-E2 conformation equilibrium was shifted towards the E2 conformation, a change resulting from alteration of both the forward and the backward reaction rate. Removal of the intracellular domain of the β subunit modifies several aspects of the whole enzyme function by a mechanism that must imply the state of the extracellular and/or transmembrane parts of the α/β subunit complex. © 1999 Elsevier Science B.V. All rights reserved.

1. Introduction

The Na,K pump is a ubiquitous membrane bound ATPase which carries sodium ions out and potassium ions into the cell and thereby maintains the Na^+ and K^+ gradients across the membrane of all animal cells. These ionic gradients are involved in a number of important cell functions like membrane excitability, cell volume regulation, and transepithelial secretion and reabsorption [1]. The Na,K pump is composed of an α and a β subunit. In the kidney a γ subunit seems also to be associated [2,3]. The α subunit is known as the 'catalytic' subunit because ATP binding and hydrolysis are performed by an intracellular domain of this protein [1]. The β subunit is known to be essential for biosynthesis, maturation and migration of the functional Na,K pump from the endoplasmic reticulum to the cell surface [4]. Moreover, this β subunit has been shown to be involved in functional aspects of the active Na,K pump, namely in the apparent affinity for extracellular K^+ [5–7].

According to the Albers-Post kinetic scheme, the Na,K pump exists alternately in two major conformations during the Na,K transport [8,9]. The E1 conformation has a high affinity for intracellular

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Na^+ which can access to its intracellular cation binding sites and the E2 conformation, which exposes high affinity sites for extracellular K^+. The activity of the Na,K pump is electrogenic since it moves 3 Na^+ out and 2 K^+ into the cell during a full transport cycle. Experimental evidence suggests that the main charge translocation occurs during the transport and release to the outside of sodium ions [10–12]. This Na^+-dependent charge translocation has allowed the study of the kinetics of single steps in the Na^+ transporting limb of the cycle, i.e. when the Na,K pump activity is studied in the Na^+/Na^+ exchange mode. Indeed, this could be done by measurements of ouabain-sensitive pre-steady-state currents following fast voltage perturbation in myocardial cells [13,14], in *Xenopus* oocytes with endogenous pump [15,16] and artificially expressed wild-type and mutant Na,K pump subunits [17].

It has been shown that truncation of the N-terminal 34 amino acids, comprising the whole cytoplasmic domain of the β subunit, induced a significant decrease in the apparent affinity of the Na,K pump for extracellular K^+ [6]. In a recent detailed work [18], it has been proposed that the N terminus is not directly involved in the K^+ effect but that truncation perturbs the ectodomain and/or the transmembrane domain of the β subunit. The mechanism of this structural perturbation remains to be explained. Functionally, it has been shown that the apparent affinity for extracellular K^+ was decreased by a factor 2–8, depending on the presence of extracellular Na^+ [18]. Moreover, the apparent affinity for intracellular Na^+ was also decreased by a factor of about 2 in Na,K pumps containing the N-terminally truncated β subunit. In order to understand how the removal of an intracellular domain of the 'accessory', non-catalytic subunit could have such a large effect on the function of the Na,K pump, and in particular on the apparent affinity for extracellular K^+, we have characterized in more detail several functional aspects of Na,K pumps containing a wild-type or a N-terminally truncated β subunit. Using the classical two-electrode voltage-clamp technique and a modified cut-open oocyte technique, we obtained evidence indicating that the β N-terminal truncation modifies several functional properties of the Na,K pump, in addition to the apparent affinities for cations, in particular the E1-E2 conformation equilibrium and the

barrier property. In accordance with recent molecular analysis [18], our results suggest that truncation of the N-terminal intracellular domain of the β subunit results in a structural modification of the transmembrane and extracellular parts of the whole enzyme.

2. Materials and methods

2.1. Generation of the N-terminally truncated mutants of the β subunit and expression of the Na,K pump in Xenopus oocytes

The production of a mutant of the (Na,K)-ATPase β isoform with a 34-amino acid N-terminal truncation (t34β mutant) has been previously reported [6]. Stage V/VI *Xenopus laevis* oocytes were obtained from ovarian tissue of females which had been anesthetized by immersion in MS 222 (2 g/l; Sandoz, Basel, Switzerland). The oocytes were defolliculated as previously described [19]. Wild-type (WT) and mutant *Xenopus* β subunit cRNA (1 ng) was injected with wild-type *Bufo* α subunit cRNA (7 ng) in a total volume of 50 nl water, into oocytes as already described [19]. The oocytes were kept in a modified Barth's solution and electrophysiological measurements were performed 3 days later. The oocytes were Na^+ loaded by a 2 h incubation in a K^+-free and Ca^{2+}-free solution containing 90 mM Na^+ and 0.5 mM EGTA. They were then kept for at least 2 h in a K^+-free solution with 0.4 mM Ca^{2+} and 200 nM ouabain [20]. These conditions allowed a selective study of the exogenous Na,K pumps composed of *Bufo* $α_1$ (ouabain resistant) and *Xenopus* $β_1$ subunits, in the presence of inhibited, endogenous ouabain-sensitive, *Xenopus* Na,K pumps [5].

2.2. Measurements of steady-state currents with the two-electrode voltage-clamp technique

We used the two-electrode voltage-clamp technique to measure the steady-state Na,K pump currents, as described earlier [5]. Voltage and current were recorded with a TEV-200 voltage-clamp (Dagan, Minneapolis, MN, USA). In each oocyte the Na,K pump current, i.e. the outward current activated by addition of 10 mM K^+ to an initially K^+-free solu-

tion, was measured at −50 mV. This value has been shown to be a good estimate of the number of Na,K pumps at the oocyte surface [21] and was used to normalize the current values in the different experimental conditions. The voltage dependence of the ouabain-sensitive currents was determined by measuring the current during a series of nine 250 ms equal voltage steps (−130 to +30 mV or −170 to +70 mV). This was done before and after the addition of 2 mM ouabain. When the effect of ouabain was studied in solutions with different pH (7.4 and 6.0), measurements were first performed in each oocyte under the two conditions in the absence of ouabain. Two mM of ouabain was then added and the same series of measurements was repeated 2 min later.

2.3. Measurements of pre-steady-state currents with the cut-open oocyte technique

In order to obtain a voltage clamp with high time resolution for the investigation of short pre-steady-state current due to transient charge movement by the pump, we used a modified cut-open oocyte technique. This technique, originally developed by Taglialatela et al. [22], has already been used for the study of the pre-steady-state current of the (Na,K)-ATPase by Holmgren and Rakowski [16]. Briefly, a *Xenopus* oocyte was mounted between two compartments with the vegetal pole upwards. The superior pole of the oocyte was in contact with the upper bath through a hole of 0.9 mm in diameter. The middle (guard) bath served to provide electrical isolation between the upper (extracellular) and lower (intracellular) compartments. This was obtained through independent voltage clamping of the middle bath at the same electrical potential as the upper one. The lower pole of the oocyte was impaled with a glass microelectrode which was simultaneously used as an internal perfusion pipette and a voltage-recording electrode. This modification to the original set-up was first described and successfully used by Costa et al. [23]. The resistance of the electrode, when filled with the intracellular solution described below, was about 0.2–0.7 MΩ. The external compartment was superfused by gravity (flow rate approx. 6 ml/min) with an extracellular Na$^+$-containing solution (see below). However, the pre-steady-state currents were measured under no flow conditions in order to reduce

the noise associated with the extracellular fluid perfusion. The internal solution was perfused by means of a precision syringe pump (Infors, Basel, Switzerland) at a flow rate of 1 μl/min. The voltage clamp was performed using a Dagan cut-open oocyte apparatus (Dagan, Minneapolis, MN, USA; Model CA-1 High Performance Oocyte Clamp). Data acquisition and analysis were performed using a TL1 DMA digital converter system and the pClamp software package (Axon Instruments, Foster City, CA, USA; version 5.5). The holding potential was −50 mV. Voltage pulses of 30 ms were made from the holding potential over the range of −190 to +90 mV in increments of 35 mV. Twenty runs of recordings were averaged. The current signal was filtered at 1 or 2 kHz using a four-pole Bessel filter. The ouabain-sensitive transient currents were obtained by subtraction of the currents measured 2 min after the superfusion of the same solution but containing 2 mM ouabain from that measured before.

2.4. Determination of Na,K pump turnover rate

The turnover rate of the wild-type and mutated Na,K pump was determined by dividing the mean K$^+$-activated outward current (in charges per second) by the mean number of ouabain binding sites determined in a group of oocytes from the same batch, as previously described [21]. These experiments were performed using the α subunit from *X. laevis* in order to allow the measurements of high affinity ouabain binding sites (ouabain binding measurements are technically difficult and prone to large errors with the ouabain-resistant *Bufo* α$_1$ isoform). Briefly, Na$^+$-loaded oocytes were exposed for 30 min to a K$^+$-free solution containing 0.67 μM cold ouabain and 0.33 μM ^{3}H-ouabain (Amersham). Oocytes were then washed and individually counted after solubilization in 100 μl of 5% SDS and addition of 2 ml of emulsifier scintillator plus scintillation liquid (Packard).

2.5. Solutions and drugs

The compositions of the solutions used for the pre-steady-state currents and pump turnover measurements were as follows: extracellular Na$^+$-containing solution (mM): Na$^+$ 92.4, Mg^{2+} 0.82, Ba^{2+} 5, Ca^{2+}
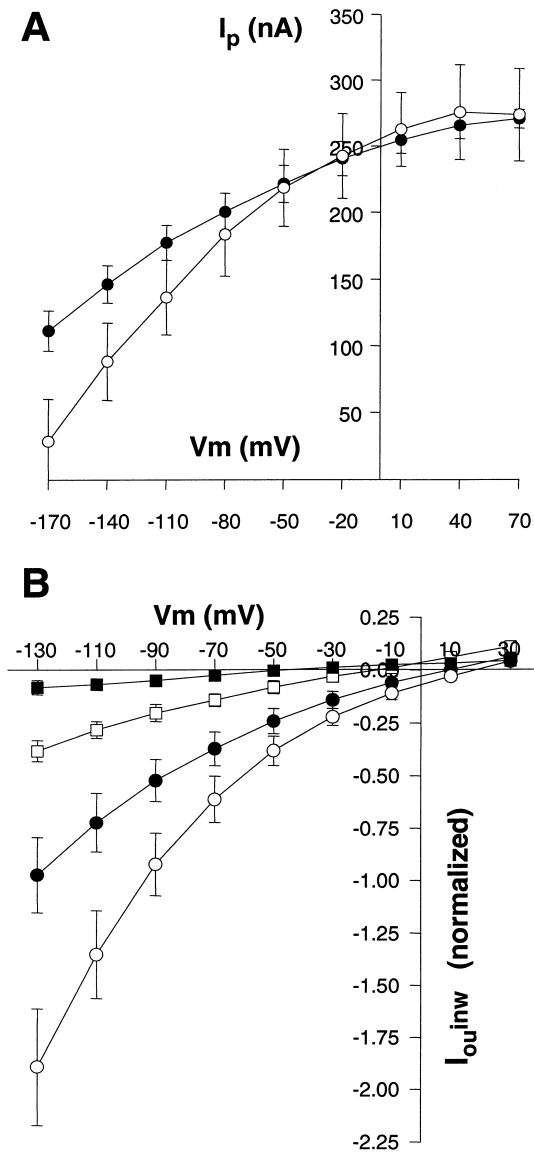
Fig. 1. Steady-state ouabain-sensitive current-voltage relationships in the presence of 10 mM K$^+$ and in K$^+$-free solution. (A) Forward Na,K pump currents were activated with 10 mM K$^+$ and ouabain-sensitive currents (I_p) were determined by subtraction of current-voltage relationships before and 2 min after addition of 2 mM ouabain in both groups: wild-type (●, $n = 8$) and β N-terminally truncated Na,K pump (○, $n = 4$). At −170 mV, I_p was significantly smaller in the β N-terminally truncated Na,K pumps as compared to the wild-type. (B) In a K$^+$- and Na$^+$-free solution at pH 6.0, a larger inward ouabain-sensitive current ($I_{ou}inw$) was observed with the β N-terminally truncated Na,K pump (○, $n = 15$) compared to the wild-type pump (●, $n = 14$). In the presence of extracellular Na$^+$ and at pH 7.4, $I_{ou}inw$ was very small in wild-type (■, $n = 5$) but clearly larger in the β N-terminally truncated Na,K pump (□, $n = 5$). The ouabain-sensitive inward currents were normalized to the 10 mM K$^+$-activated Na,K pump current at −50 mV.

0.41, tetraethylammonium (TEA) 10, Cl$^-$ 22.5, HCO$_3^-$ 2.4, gluconate 80 and HEPES 10, pH 7.4; solution for the intracellular perfusion: Na$^+$ 50, K$^+$ 50, Mg^{2+} 0.82, gluconate 100, Cl$^-$ 1.64, Tris-adenosinetriphosphate (ATP) 5, EGTA 2, pH 7.35. For the pH-dependent ouabain-sensitive current experiments the 'control' K$^+$-free solution contained Ba^{2+} 5, TEA 10, Mg^{2+} 0.82, Ca^{2+} 0.41, (N-morpholino)propanesulfonic acid (MOPS) 53, Cl$^-$ 22, sucrose 170. The pH was adjusted to 7.4 with N-methyl-D-glucamine (NMDG). Ten mM K$^+$ was added as K-gluconate (osmolarity was not compensated for) for activation of the Na,K pump. Ouabain was obtained from Sigma (St. Louis, MO, USA). All the experiments were performed at room temperature (22–27°C). Results are expressed as mean ± S.E.M., n = number of tested oocytes if not stated otherwise. The statistical significance of differences between means was estimated using bilateral Student's t-test for unpaired data.

3. Results

3.1. Steady-state current in the wild-type and β N-terminally truncated Na,K pump

As already observed by Hasler et al. [18], using the two-electrode voltage-clamp technique, the mean ouabain-sensitive steady-state Na,K pump current (I_p) (activated with 10 mM K$^+$ at −50 mV) was similar for the wild-type (222 ± 19 nA, $n = 8$) and β N-terminally truncated (219 ± 29 nA, $n = 4$) Na,K pumps. Fig. 1A shows the current-voltage relationships of the ouabain-sensitive current of both Na,K pumps. At large negative membrane potential values ($< −150$ mV), I_p tended to be smaller in the β N-terminally truncated compared to the wild-type Na,K pumps. This difference was statistically significant ($P < 0.05$) at −170 mV: 112 ± 15 nA ($n = 8$) for the wild-type Na,K pump versus 29 ± 32 nA ($n = 4$) for the β N-terminally truncated Na,K pump.

3.2. Ouabain-sensitive inward-rectifying current ($I_{ou}inw$)

To further characterize the biophysical properties of the mutant pump, we studied the ouabain-sensi-

tive inward current (I_{ou}inw) which can be measured with extracellular Na$^+$- and K$^+$-free solutions [17,24,25]. The amplitude of this current is larger with extracellular solutions of low pH and it has been proposed to be an inward proton current flowing through the E2 conformation of the Na,K pump [25]. With an extracellular solution of pH 6.0, which increases the I_{ou}inw, the normalized inward current was about twice larger (at −130 mV) for the β N-terminally truncated as compared with that for the wild-type Na,K pump (Fig. 1B and Table 1). The amplitude of I_{ou}inw was smaller in both groups at neutral extracellular pH, but it was still about 3 times larger in the β N-terminally truncated than in the wild-type Na,K pumps (Table 1). We further measured the ouabain-sensitive current in the presence of extracellular sodium (92 mM) at pH 7.4, a condition in which almost no ouabain-sensitive conductance can be detected [5]. We could still observe a sizeable I_{ou}inw in the β N-terminally truncated mutant Na,K pump which, at −130 mV, reached about one third of the forward Na,K pump (K$^+$-activated) current at −50 mV (Fig. 1B and Table 1). With the wild-type pump this current was reduced to less than one tenth of the forward current (Table 1).

3.3. Transient currents in the Na$^+$/Na$^+$ exchange mode

In the absence of extracellular K$^+$ and with high concentrations of Na$^+$ on both sides of the membrane, the (Na$^+$+K$^+$)-ATPase functions in the so-called Na$^+$/Na$^+$ exchange mode [26]. For this exchange, we can use the simplified reaction scheme [13]:

$$Na_i + E1 \rightleftarrows E2 + Na_o.$$
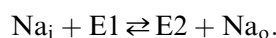
It has been shown that during this exchange, charges are translocated across the membrane inducing measurable transient (pre-steady-state) currents when the membrane potential is rapidly changed [13,15–17]. A simple model has been proposed to explain this observation: the transport of three sodium ions out of the cell and the associated E1 to E2 conformational change result in the translocation of one net charge across the membrane electrical field. Thus, the time course of the transient current relaxation reflects the redistribution of the E1 and E2 states towards the new equilibrium imposed by the membrane potential. Because no transient current could be detected in the K$^+$/K$^+$ exchange conditions [27], a simple model has been proposed in which the translocation of two potassium ions is electroneutral because during the translocation, these ions are associated with two negative charges of the protein while a transfer of one net charge occurs when three sodium ions are transported across the membrane, two of the three sodium ions being associated with the protein negative charges. Further results have shown that the charge translocation mechanism may be more complicated with several components of different time course [14,28,29] and that the transfer of K$^+$ may also be accompanied by charge translocation [30]. In our experimental conditions, using the cut-open oocyte technique, the fast applied voltage steps caused large transient currents mainly due to the capacitance of the oocyte membrane (Fig. 2A,B). The pre-steady-state currents due to the Na,K pump were obtained by subtracting the current values before and after ouabain application. Fig. 2A and B show original

Table 1
Ouabain-sensitive inward current in K$^+$-free solutions as a function of extracellular pH and Na$^+$ concentration

I_{ou}inw at −130 mV (normalized)	β wild-type	β N-terminally truncated
pH 6.0, Na$^+$ 0 mM	0.97 ± 0.18	1.89 ± 0.28*
	$n = 12$	$n = 15$
pH 7.4, Na$^+$ 0 mM	0.44 ± 0.07	1.23 ± 0.26**
	$n = 10$	$n = 8$
pH 7.4, Na$^+$ 92 mM	0.08 ± 0.03	0.38 ± 0.05*
	$n = 5$	$n = 5$

All values were normalized to the maximal K$^+$ activated outward current measured immediately before in the same oocyte.
* and ** indicate mean values significantly different from that of the wild-type group ($P < 0.05$ and 0.01 respectively); n, number of observations.
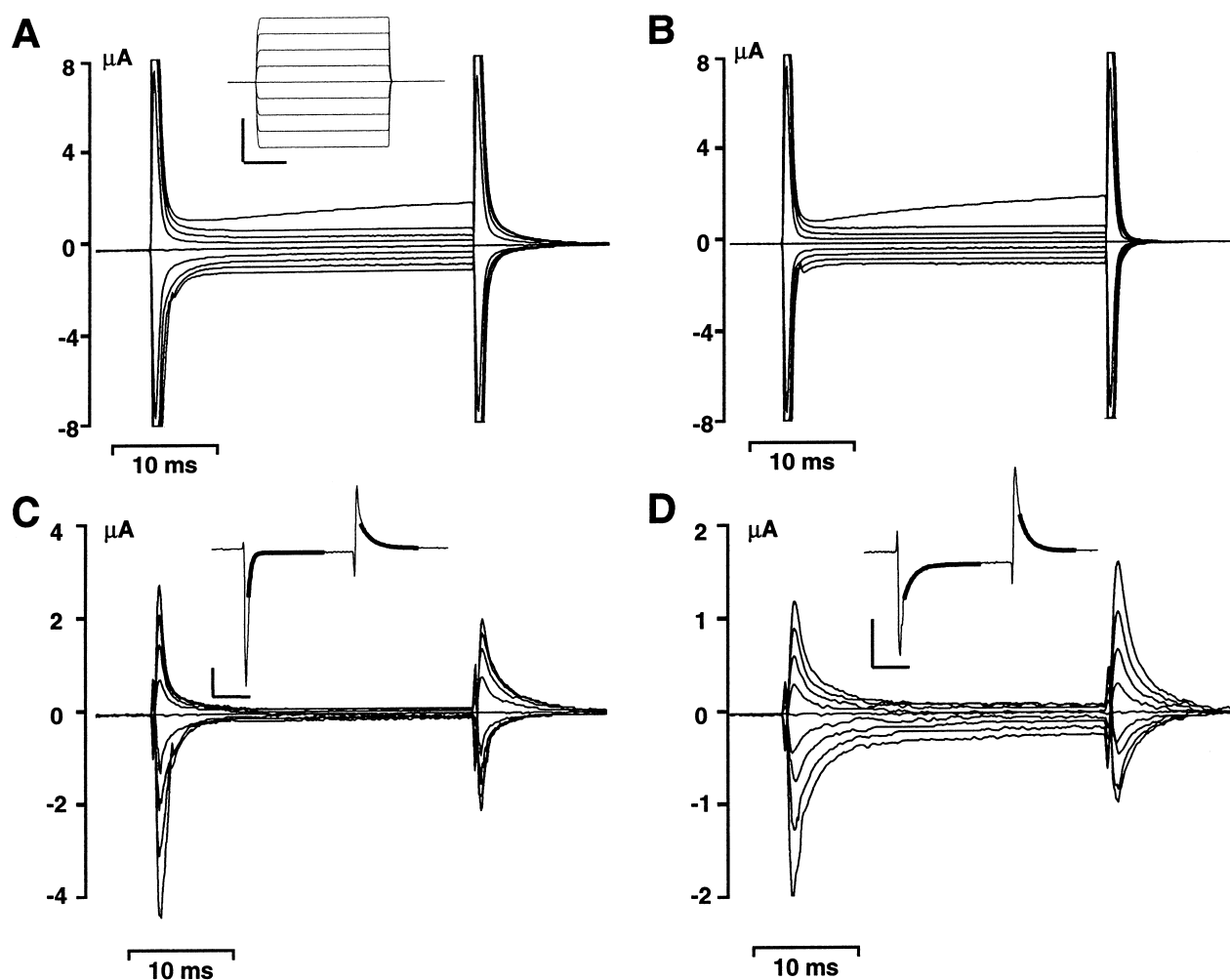
Fig. 2. Original recordings of pre-steady-state currents obtained using the cut-open oocyte technique. (A,B) Original recordings of the total current across the oocyte membrane patch (0.9 mm of diameter) recorded before (A) and 2 min after (B) addition of 2 mM ouabain in an oocyte expressing the wild-type Na,K pump. The inset in A shows a recording of the corresponding voltage traces during the series of 30 ms voltage steps from -190 to $+90$ mV; the calibration bars are 10 ms and 100 mV. The ouabain-sensitive currents (C) were obtained by subtraction of currents in A and B. Panel D shows an example of ouabain-sensitive currents in an oocyte expressing the β N-terminally truncated Na,K pump; the presence of an inward steady-state current can be noticed. For both types of pump, the 'on' and 'off' current relaxations starting 2 ms after the membrane voltage change could be well fitted to a monoexponential decrease as illustrated in insets in C and D. Calibration bars are 10 ms and 1 μA for both insets.

recordings of the voltage and current traces before and after addition of 2 mM of ouabain to the extracellular solution. Using the cut-open oocyte technique, a stable membrane voltage was obtained after about 1 ms (Fig. 2A, inset). The ouabain-sensitive current component is easily seen when the tail currents in both conditions are compared. Fig. 2C and D show the presence of an ouabain-sensitive transient current observed in oocyte expressing wild-type and β N-terminally truncated Na,K pumps. About 2 ms after the command voltage step, the transient

current decreased with a relaxation rate which could be fitted with a mono-exponential function (insets in Fig. 2C,D). The current in the first 2 ms was too variable between the individual preparations and was not considered for analysis. We therefore restricted our measurement to the slow component of the ouabain-sensitive pre-steady-state current, as also done in previous work [17].

Similarly to what has been described above about the results obtained using the two-electrode voltage-clamp technique (Fig. 1B), we also observed using

the cut-open oocyte technique that very little steady state ouabain-sensitive current could be detected in the wild-type Na,K pump (Fig. 2C) while we observed a sizeable ouabain-sensitive steady-state current in the β N-terminally truncated mutant (Fig. 2D).

3.4. Ouabain-sensitive charge translocation

The quantity of charge moved during the 'on' (Q_{on}) and the 'off' (Q_{off}) phases of the voltage step were, in a first analysis, calculated using the integration routine of the pClamp program between 0 and 20 ms after the start of the voltage change. Because of the presence of an ouabain-sensitive steady-state current with the β N-terminally truncated mutant (see above), the amount of charge due to the steady-state component was subtracted from the total current integration to estimate true Q_{on}, i.e. the charge component due to the transient current only. As shown in Fig. 3A (wild-type) and B (β N-terminally truncated mutant) the absolute values of Q_{on} and Q_{off} were similar. The mean values of the ouabain-sensitive charge displacement (Q_{ou}) were calculated from $-Q_{off}$ to avoid errors due to the steady-state current in β N-terminally truncated Na,K pump. Fig. 4A shows the Q_{ou}/V_{m} relationships for

the wild-type and mutant Na,K pump. For the wild-type Na,K pump the relationship was sigmoidal and could be well fitted with the Boltzmann equation:

$$Q_{ou} = Q_{min} + Q_{max}/(1 + \exp[z_{q}(V_{q}-V_{m})(F/RT)])$$
(1)

where F is the Faraday constant, R is the Boltzmann constant, T is the temperature, Q_{max} is the maximal displaceable charge, V_{q} is the midpoint potential, z_{q} a steepness factor and V_{m} the membrane voltage. The values of the parameters of the Boltzmann equation (see legend of Fig. 4) obtained from the mean data of the wild-type Na,K pump group were in reasonable agreement with those obtained by us and others using various techniques considering that, in these experiments, only about one third of the total membrane surface was studied [13,15–17].

The shape of the Q_{ou}/V_{m} relationship was different for the β N-terminally truncated mutant Na,K pumps. As shown in Figs. 3B and 4A, the curve did not present the characteristic sigmoidal shape as it did not saturate at negative potential values in the explored voltage range. Attempts to fit the parameters of the Boltzmann equation to this curve yielded a much reduced steepness factor (about 3–4 times smaller than for wild-type pumps, see Fig. 4A),
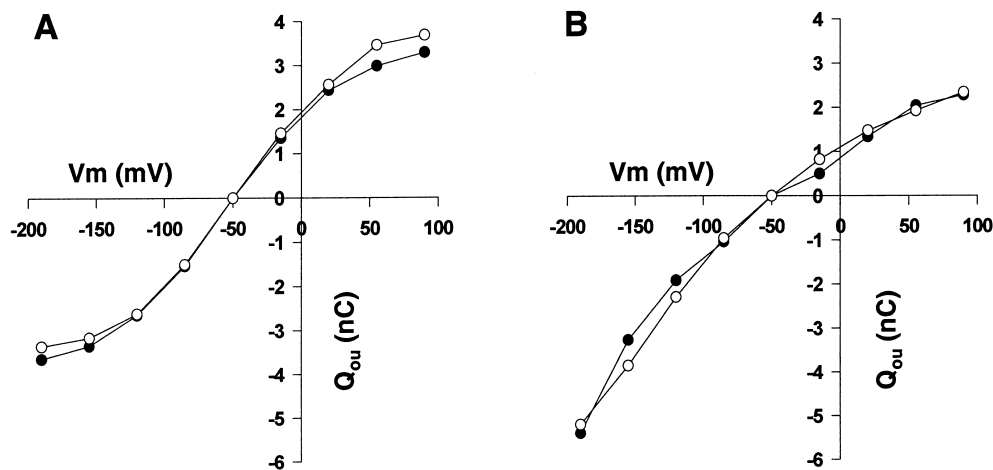

Fig. 3. Ouabain-sensitive charge translocation Q_{on} and Q_{off} with exogenous expressed wild-type and β N-terminally truncated pumps in typical experiments using the cut-open oocyte technique. (A) The charge translocation Q_{on} (●) and $-Q_{off}$ (○), calculated as time integral of the pre-steady-state current vs. the membrane potential, is represented for the wild-type pump. There was no difference in absolute values between the Q_{on} and Q_{off}. The relationship shows saturation at high negative and positive potentials. (B) For the β N-terminally truncated mutant Na,K pump, the Q_{on} (●) were identical to the $-Q_{off}$ (○) provided that the amount of charge due to the steady-state current component was subtracted from Q_{on} (see text). Compared to the wild-type relationship, the Q-values showed no tendency to saturation at high negative potentials.
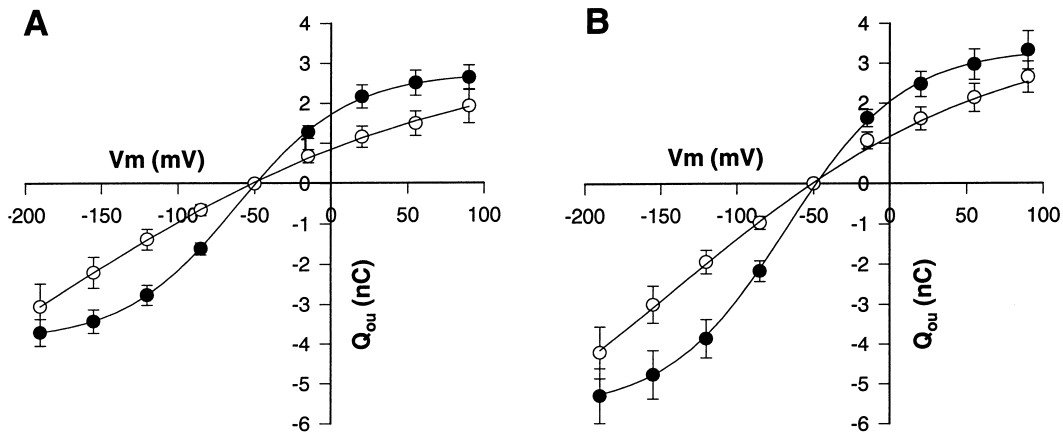
Fig. 4. Ouabain-sensitive charge translocation (Q_{ou}) as a function of membrane voltage using two modes of Q_{ou} estimation. (A) The charge translocation, obtained as time integral of the recorded current, is represented as a function of membrane voltage. The curves are drawn using the best fitting parameters from Eq. 1 on mean values. For the wild-type Na,K pump (●, $n = 17$) and β N-terminally truncated mutant (○, $n = 19$), the values of Q_{max} (maximal translocated charge) were 6.6 and 12.6 nC, respectively, the values of z_q (steepness factor) 0.71 and 0.19, respectively and the values of V_q (midpoint potential), −62 and −192 mV, respectively. (B) Slow component of the ouabain-sensitive charge displacement estimated from the monoexponential current relaxation between 2 and 20 ms (see text). Curves are drawn using the best fitting parameters from the Boltzmann equation on mean values. For the wild-type group (●, $n = 17$) and β N-terminally truncated mutant (○, $n = 17$), the Q_{max} were 9.0 and 12.9 nC, the z_q 0.68 and 0.25 and the V_q −68 and −142 mV, respectively.

a more negative value of midpoint potential for the mutant compared to that of the wild-type Na,K pumps and a larger maximal displaceable charge (Q_{max}). The mode of estimation of the ouabain-sensitive displaced charge using current integration includes in theory all the components (fast and slow) of the charge transfer. However, due to the uncertainty and the variability of the very initial currents (first 2 ms), this mode of calculation may be prone to errors. We therefore also analyzed specifically the charge translocation due to the slow component by calculating the integral over time of the ouabain-sensitive current, assuming an exponential decrease in this current with a rate constant K_{on}:

$$I = I(0) \exp(-K_{on}\, t) \tag{2}$$

Using the values of K_{on} obtained from the best fitting monoexponential to the current decay between 2 and 20 ms after the voltage step and the amplitude of the current at time 2 ms current values, we calculated Q_{ou} as the integral over time of Eq. 2 extrapolated from $t = 0$ to ∞. These Q_{ou} values are presented on Fig. 4B. The best fitting parameters of the Boltzmann equation were obtained for the set of $Q_{ou}(V)$ values obtained in each oocyte. The mean values of these parameters were respectively for the wild-type

($n = 17$) and the mutant ($n = 17$) groups: Q_{max} 9.1 ± 1.2 and 14.5 ± 2.2 nC, $P < 0.05$, z_q 0.73 ± 0.07 and 0.28 ± 0.03, $P < = 0.001$, and V_q −69 ± 2 and −172 ± 28 mV, $P < 0.001$.

It has to be noted that because the range of our data covered only a part of the sigmoidal curve in the case of the mutant Na,K pump, the values of these parameters should be taken with care. But, despite this reservation, two differences are striking: the low slope of the curve indicates that the voltage dependence of the charge translocation is reduced and the large shift to the left of the Q_{ou}/V_m relationship suggests that the mutant Na,K pump is strongly shifted towards the E2 conformation at physiological membrane potentials.

3.5. The relaxation rates of the ouabain-sensitive currents

The relaxation rates of the 'on' tail current (k_{on}) and 'off' tail current (k_{off}) were determined by fitting a single exponential equation to the current values starting 2 ms after the potential change. Over this time frame, the fit was good (see Fig. 2C,D, insets), and could not be significantly improved with a second order exponential equation. The mean values of
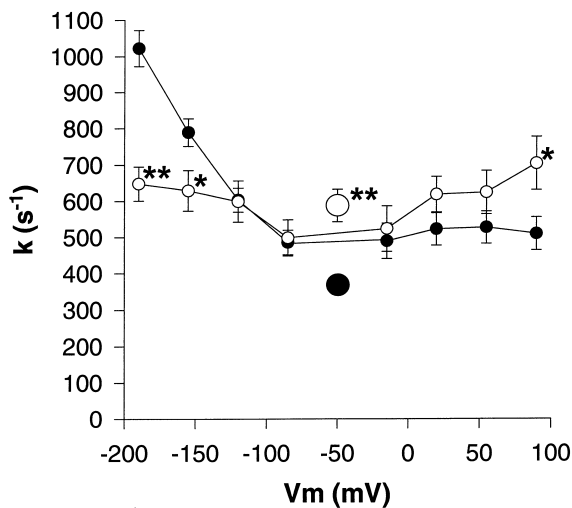
Fig. 5. Voltage dependence of the relaxation rate constants (k) of the pre-steady-state ouabain-sensitive current. The mean 'on' relaxation rate (at the beginning of the voltage step) is shown as a function of voltage. Wild-type ($n = 22$) and β N-terminally truncated ($n = 18$) values are represented by filled and open circles, respectively. The mean 'off' relaxation rate (upon return to −50 mV at the end of the voltage step) is given by the large symbols at −50 mV. Note the strong voltage dependence of the k_{on} values for the wild-type Na,K pump at high negative membrane potentials compared with the low voltage dependence of the β N-terminally truncated mutant Na,K pump. * and ** indicate values significantly different from that of the wild-type group ($P < 0.05$ and 0.01 respectively).

k_{on} for both groups expressed as a function of voltage are shown in Fig. 5. The mean values of k_{off}, which were similar for all voltage steps, are indicated by the large symbols at the −50 mV point. For the wild-type Na,K pump, k_{on} showed a clear voltage dependence with rates larger at negative potentials. In comparison, the k_{on} of the β N-terminally truncated Na,K pump was almost independent of voltage. At negative potential values, the relaxation rates were significantly smaller in the mutant group and at positive values, they were significantly higher (Fig. 5 and Table 2). Because of the large shift of the Q_{ou}/V_m curve towards negative potentials, the absolute value of charge translocation after voltage step from −50 mV to more positive potentials was small in the β mutant Na,K pumps, resulting in a small signal to noise ratio. In order to examine the voltage dependence of the relaxation rate constant with a large signal and obtain more precise measurements, we used the following pulse protocol: from the holding potential, a pre-pulse of 35 ms at −150 mV was

followed by a test pulse to +50 mV. As shown in Table 2, the mean K_{on} obtained from −150 to +50 mV voltage jumps was about twice faster with the β N-terminally truncated than with the wild-type Na,K pumps.

3.6. Turnover rates of the wild-type and β N-terminally truncated Na,K pump

The estimation of Q_{max} was larger with the β N-terminally truncated Na,K pump than with the wild-type Na,K pump (Fig. 4), while there was no significant difference in the steady-state Na,K pump current [18]. This observation could be explained by a slower turnover rate of the β N-terminally truncated Na,K pump if expression of these mutants resulted in a higher density of Na,K pumps. We therefore determined the turnover rate in the two groups by dividing the mean steady-state pump current by the mean number of ouabain binding sites for a given batch of oocytes. For these experiments, we injected the *Xenopus* α subunit, instead of the ouabain-resistant *Bufo* subunit, in order to be able to perform the ouabain binding experiment. There was no significant difference between the two groups either for the pump current (wild-type 436 ± 61 nA and β N-terminally truncated 327 ± 88 nA, $n = 3$ batches of oocytes with 6–10 oocytes per batch) or for the number of ouabain binding sites (wild-type 48.5 ± 3.9 × 10⁹ sites/oocyte and β N-terminally truncated 47.4 ± 5.6 × 10⁹ sites/oocyte). The calculated turnover rates for the wild-type and β N-terminally truncated Na,K pumps were 55 ± 3 and 44 ± 12 charges/s, respectively. For the endogenous *Xenopus*

Table 2
Relaxation rate constant (k_{on}) of the pre-steady-state ouabain-sensitive current

k_{on} (s⁻¹)	β wild-type	β N-terminally truncated
from −50 to −190 mV	1022 ± 50	648 ± 47**
	$n = 22$	$n = 18$
from −50 to +90 mV	510 ± 46	704 ± 73*
	$n = 22$	$n = 18$
from −150 to +50 mV	313 ± 14	573 ± 21**
	$n = 11$	$n = 9$

* and ** indicate mean values significantly different from that of the wild-type group ($P < 0.05$ and 0.01 respectively).

pump, i.e. in non-injected oocytes, the current was about only one tenth of the exogenous one, but the turnover was also in the same range (59 ± 12 charges/s).

4. Discussion

Among the large family of the P-type ATPases, the (Na,K)-ATPase together with the gastric and non-gastric types of (H,K)-ATPase form a distinct group of ion pumps. All known members of this group carry out the transport of potassium ions inside the cell in exchange for the extrusion of Na^+ or H^+ out of the cell. One common and specific characteristic of this subgroup of P-ATPase is the presence of a β subunit associated with the main 'catalytic' α subunit. The presence of this β subunit seems to be absolutely required for the expression of a cation transport function by this group of proteins (for a review see [7]), although the α subunit alone may be able to perform ATP hydrolysis [31].

While the role of the β subunit in the maturation process has been well documented [32], its role in the mature enzyme expressed at the cell surface is not as well understood. Functional studies in several expression systems have shown that the type of the β subunit that is associated with the α subunit influences the function of the whole enzyme (for review see [7]), in particular its apparent affinity for extracellular K^+ [5]. Although the three main domains (intracellular N-terminal, transmembrane and extracellular C-terminal domains) of the β subunit have been implicated in this effect, the mechanism of the α/β subunit interaction is still poorly understood [6,32]. Hasler et al. [18] have recently shown that a truncation of the whole N-terminal domain of the β subunit induces a large reduction of the apparent affinity for extracellular K^+ and a smaller reduction of the apparent affinity for intracellular Na^+. In the present work, we have explored in more detail the effects of the N-terminal truncation of the β subunit on the function of the Na,K pump.

As recently reported [18], we found that the β N-terminally truncated Na,K pump was able to associate with the α subunit and to form a fully functional Na,K pump at the oocyte surface. The maximal rate of cation transport estimated from the ratio between the K^+-activated Na,K pump current (under V_{max} conditions) to the number of ouabain binding sites was similar in wild-type Na,K pumps and pump containing the N-terminally truncated β subunit. The current-voltage relationship of the ouabain-sensitive current was only slightly altered (see Fig. 1A), with a small increase in the voltage dependence of this current at low membrane potentials. These observations suggest that the rate-limiting transport reactions were little altered by the N-terminal truncation of the β subunit. More detailed measurements revealed, however, that the Na,K pump function was nevertheless profoundly modified.

A striking difference between wild-type and β N-terminally truncated Na,K pumps was the presence of an ouabain-sensitive inward current when the mutant was studied in the absence of external K^+, a condition in which no Na/K exchange can occur. The role of Na,K pumps is to establish and maintain the gradients of Na^+ and K^+ across the plasma membrane. An efficient ion pump function requires that little leak of ions occurs, otherwise energy (ATP) would be spent with no gain. Indeed, the normal (Na,K)-ATPase is very 'tight'. Under conditions in which no active transport occurs, such as the absence of extracellular K^+, hardly any ouabain-sensitive ion conductance can be detected [5], except for the pH-sensitive conductance when the measurements are performed in the absence of extracellular Na^+ and K^+ [25], i.e. under far from physiological conditions. As shown in Fig. 1B, the Na,K pump containing a β N-terminally truncated subunit showed a significant 'leak', demonstrated by the presence of an ouabain-sensitive conductance at a physiological pH and external Na^+ concentration. The amplitude of the pH-sensitive conductance was also significantly increased in the β N-truncated mutant. The steeper I/V curve of the steady-state ouabain-sensitive current observed in the presence of external K^+ suggest that the 'leak' conductance is also present in the presence of K^+. This hypothesis, however, would be difficult to demonstrate, because we have no easy way to distinguish which part of the total ouabain-sensitive current is due to the forward Na/K exchange cycle and which part could be due to a 'leak' conductance.

The transport of three sodium ions out of the cell, and the associated E1 to E2 conformation change, are accompanied by the translocation of one net

charge across the membrane electrical field [13,33]. More precisely, the main charge translocating event seems to be associated with the deocclusion and release of the first sodium ion to the outside medium [14]. This charge movement can be measured as a pre-steady-state ouabain-sensitive current following rapid membrane voltage perturbations under conditions of Na^+/Na^+ exchange. With the wild-type Na,K pump, the voltage dependence of the charge translocation can be well described by a Boltzmann function, as shown in several previous reports [13,16,17] and by our results (Fig. 4), with a steepness factor z of about 0.75. In contrast, in the β N-terminally truncated Na,K pump, the voltage dependence was much reduced and no saturation could be observed at membrane potentials as negative as −190 mV. The best fitting parameters of the Boltzmann equation indicated a larger maximal displaceable charge. Since no increase in the density of ouabain binding sites could be detected when the *Xenopus* α_1 subunit was expressed with the N-truncated β subunit (when compared with the wild type β subunit expressed with the same α subunit), these results suggest that the modified Na,K pump carries out a larger maximal charge displacement upon voltage perturbation. Whether this change is related to a redistribution between the fast and slow components of the charge-translocating events or whether it due to the presence of the movement of additional intrinsic charges in the membrane electrical field cannot be determined by our present results. In any case, these results have to be regarded with caution since rather large errors could be made in the estimation by extrapolation of the maximal charge displacement because of the low slope of the Q_{ou}/V_m relationship.

The midpoint potential, which had a value of about −60 mV in wild-type Na,K pump, was displaced toward much more negative potentials in the β N-truncated pumps. This suggests that at −60 mV the β N-truncated Na,K pump is mostly in the E2 conformation, while the wild-type Na,K pump is nearly equally distributed between the E1 and the E2 conformation. This interpretation is supported by analysis of the kinetics of the charge translocation (Fig. 5) which show a slower inward (E2 → E1) and a faster outward (E1 → E2) charge translocation in the mutant Na,K pump.

Taken together, with the earlier published results obtained with the same mutant showing a 2–8-fold reduction in apparent affinity for extracellular K^+ (depending on the presence or absence of extracellular Na^+) and an approx. 2-fold reduction in apparent affinity for intracellular Na^+ [18], our results indicate that the N-terminal truncation of the β subunit produces complex and profound changes in the cation transport function of the Na,K pump, even though these changes have little effect on the Na,K pump activity maximally stimulated by extracellular K^+.

In a previous study, using a simple kinetic model of the Na,K pump transport cycle [17], we have shown that N-terminal truncation of the α subunit induces changes in the apparent affinity for extracellular K^+ that could be well explained by a modification of the rate of the E1 → E2 conformation change. Using the same kinetic model we also attempted to reproduce the values for apparent Na^+ and K^+ affinities observed in the β N-terminally truncated Na,K pump by applying the values of the rate of conformation change deduced from the charge translocation measurements. However, in the case of the N-terminally truncated β subunit, we were not able to explain the large change in the apparent affinity for extracellular K^+ only by modification of the E1 ↔ E2 conformation equilibrium; a modification of the intrinsic affinity for extracellular K^+ was necessary.

We conclude that removal of the intracellular N-terminal domain of the β subunit induces significant changes in the conformation of the whole α/β complex, including modifications of the external K^+ binding site, changes in the E1 ↔ E2 conformational equilibrium and formation of a 'leaky' E2 conformation. This suggests that the state of the intra-membrane part at least, and possibly also of the extracellular part of the whole α/β complex, is dependent on the integrity of the intracellular N-terminal domain of the β subunit. This conclusion is in good agreement with those of Hasler et al. [18] who suggest that functionally important α/β subunit interactions occur between the extracellular domains of these subunits rather than between the intracellular domains. The absence of the N-terminal domain of the β subunit appears to entail important modifications of the whole α/β complex.

Acknowledgements

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